Remarks

A substitute specification and substitute declaration are filed herewith. The specification has been amended to remove the embedded hyperlink on page 5, and to capitalize the trademarks appearing on page 71.

Upon entry of this amendment, claims 1-8 and 10-15 will be pending. Claims 9, 16 and 17 have been cancelled and claims 1, 3, 4-8, and 12-14 have been amended herein. Support for the amendment to claim 1 can be found in originally filed claims 7-9. Support for the amendment to claim 3 can be found in originally filed claim 5 and the specification at, *e.g.*, page 8, lines 12-14. Support for the amendment to claim 5 can be found in Table 1 at page 6. Support for the amendments to claims 6 and 14 can be found in originally filed claim 1. Support for the amendments to claims 7 and 8 can be found in originally filed claims 1 and 7 and the specification at, *e.g.* page 35, lines 25-29, No new matter has been added.

Oath/Declaration

The Examiner has indicated that the oath or declaration is defective. A substitute Declaration executed by inventor Ferenc Boldog is provided herewith.

Specification

The Examiner has indicated that the substitute specification provided in Paper No. 4 lacks pages 12-21. A substitute specification containing pages 1-123 is filed herewith.

The Examiner has objected to the disclosure for containing an embedded hyperlink. The specification has been amended herein to remove the embedded hyperlink.

The Examiner has noted that the specification contains trademarks. The specification has been amended herein to capitalize these trademarks appearing on page 71 of the instant specification.

These objections have been overcome and can be withdrawn.

Claim Objections

The Examiner has objected to claim 14 under 37 CFR §1.75(c) as being in improper form for not refering to other claims in the alternative. Claim 14 has been amended herein to delete

the reference to claim 1. As such, claim 14 properly depends on claim 11 only. This objection should be withdrawn.

The Examiner has also objected to claims 3, 4, and 7-9 under 37 CFR §1.75(c) as being in improper dependent form for failing to limit the subject matter of claim 1, the Examiner stating "[c]laims 3, 4, 7, 8, and 9 are directed to a complement of the nucleic acid of claim 1 and, therefore, claims 3, 4, 7, 8 and 9 can be infringed by a nucleic acid, which does not infringe claim 1." Claim 9 has been canceled herein. This objection is therefore moot in regard to this claim. Claims 7 and 8 have been amended herein to delete the term "complement." Regarding claims 3 and 4, claim 1 has been amended to recite in part "or the complement of said nucleic acid sequence." Therefore, as the claims stand, claims 3, 4, 7 and 8 further limit the subject matter of claim 1, and, thus, are in proper dependent form. This objection should be withdrawn.

The § 101 rejections

Claims 7 and 8 have been rejected under 35 USC § 101 as directed to non-statutory subject matter for their use of the phrase "oligonucleotide sequence." Applicants have amended claims 7 and 8 herein to recite the phrase "[a]n isolated nucleic acid" and delete the term "oligonucleotide sequence." Therefore, Applicants assert that claims 7 and 8 are directed to statutory subject matter. This rejection has been overcome and should be withdrawn.

Claims 1-13 have been rejected under 35 U.S.C. § 101 as not being supported by an apparent or disclosed specific and substantial credible utility. (Office Action at p. 5). Claim 9 has been canceled herein. This objection is therefore moot in regard to this claim. Applicants traverse this rejection to the extent it applies to pending claims 1-8 and 10-15.

Applicants respectfully assert that the nucleic acids of the present invention have a specific, substantial, and credible utility, and therefore are patentable under 35 U.S.C. §101. Applicants assert that SEC1/FGF (clone identification number FGF10AC004449, also refered to herein as CG54455-06) can be used, *inter alia*, for detection of inflammatory diseases including psoriasis, Crohn's Disease (a disease characterized by, *e.g.*, inflammation of the bowel), and for indentification of cell proliferative disorders including cancer, and that the nucleic acid or the protein of clone FGF10AC004449 may be a target for therapeutic agents in such disorders. *See* specification at page 10, lines 25-28; and page 105, lines 15-27.

The SEC1/FGF nucleic acid and polypeptide of the present invention are characterized as members of the fibroblast growth factor family of proteins (FGFs). *See* specification at page 9, line 30 to page 10, line 21. The Utility Examination Guidelines state that "when a patent application claiming a nucleic acid asserts a specific; substantial, and credible utility, and bases the assertion upon homology to existing nucleic acids or proteins having an accepted utility, the asserted utility must be accepted by the examiner unless the Office has sufficient evidence or sound scientific reasoning to rebut such an assertion." Fed. Reg., Vol. 66. No. 4, January 5, 2001, p. 1096. If the Examiner has sufficient evidence to rebut such an assertion, and rejects the claims for lack of utility, then the burden shifts back to the applicant to provide evidence supporting such a well-established utility.

Further, one of ordinary skill in the art accepts structural homology based on amino acid sequence identity as a credible method of determining the function of a polypeptide. *See* Henikoff et al., Science, 278:609-614 (1997). Accordingly, based on the evidence presented in this response that SEQ ID NO 2 shares a high degree of homology with members of the FGF family of proteins, one skilled in the art would assign a function to SEQ ID NO 2 based on the function of those polypeptides with which it shares great homology. The FGF family of mitogens includes at least 22 members that are structurally related and share a conserved central region.

SEQ ID NO 2 shares sequence identity with various members of the human FGF family, as provided in the as-filed specification at page 9, lines 22-26. The polypeptide of SEQ ID NO: 2 is 100% identical to the human FGF-22 precursor protein, and has 79 of 145 residues (54%) identical to, and 112 of 145 residues (77%) positive with, the human FGF-10 precursor protein.

The Utility Examination Guidelines further state that "when a class of proteins is defined such that the members share a specific, substantial, and credible utility, the reasonable assignment of a new protein to the class of sufficiently conserved proteins would impute the same specific, substantial, and credible utility to the assigned protein." Fed. Reg., Vol. 66. No. 4, January 5, 2001, p. 1096. FGFs have been increasingly implicated to play important roles in inflammation, cell proliferative disorders including cancer, blood vessel formation, and arthritis. Accordingly, members of the FGF family share a specific, substantial, and credible utility, as a marker for chronic and acute inflammatory diseases, cancers, and arthritis. Moreover, the

sequences of such members are sufficiently conserved, thereby imputing the same utility to a novel member of their protein class, such as SEQ ID NO 2.

In support of Applicants' assertions, filed herewith is a Declaration under 37 C.F.R. § 1.132, by Meera Patturajan, Ph.D., an employee of CuraGen Corp., the assignee of the instant application. As noted by Dr. Patturajan, the SEC1/FGF nucleic acid and polypeptide can be used in therapeutic and diagnostic applications in inflammatory disorders such as psoriasis, and cell proliferative disorders, including cancer. (See Patturajan Declaration, ¶ 8).

As described by Dr. Patturajan, the data in Exhibit A demonstrates that the SEC1/FGF polypeptide induces proliferation in a dose-dependent manner, which is a vital function of fibroblast growth factors. (See Patturajan Declaration, ¶ 6). The teachings of the specification are consistent with the knowledge of one of skill in the art regarding expression of FGFs. (See Patturajan Declaration, ¶ 5). Figures 1a and 1b of Exhibit A demonstrate the expression and purification of the SEC1/FGF polypeptide (referred to as CG54455-06). Figure 2 demonstrates that conditioned media from a fusion protein comprising the SEC1/FGF polypeptide of SEQ ID NO: 2 induces proliferation of Baf3r2b cells (Baf3 cells expressing the FGFR-2b receptor polypeptide). Also, Figures 3 and 4 demonstrate that the SEC1/FGF fusion polypeptide and the mature form of the SEC1/FGF polypeptide induce Baf3r2b cell proliferation in a dose-dependent manner. Figure 5 demonstrates that the SEC1/FGF polypeptide induces cell proliferation specifically in Baf3 cells expressing a specific FGF receptor, FGFR-2b. Applicants note that it is known to one of ordinary skill in the art that regulation of proliferation is a key event in wound healing and a role for FGF-22 (SEQ ID NO: 2) has been recently suggested in cutaneous development and repair (See Beyer et al., Experimental Cell Research, 287: 228-236, 2003, courtesy copy enclosed herewith).

Dr. Patturajan also describes how the data in Exhibit B depict the scaled results of real time quantitative polymerase chain reaction-based gene expression analyses performed using SEC1/FGF gene-specific primer-probe set to measure the relative SEC1/FGF expression levels in normal cells or tissues, and pathological tissue samples. The AG4346 and AG4347 primer sets used are found in Tables 1 and 2. As shown in Tables 3 and 4, the Relative Expression Score for each sample indicates the relative quantity of a SEC1/FGF transcript, with 0.0 indicating no detectable expression and 100.0 indicating highest detectable expression level.

These results demonstrate that SEC1/FGF is consistently under-expressed in both male and female psoriasis patients (See Patturajan Declaration, ¶ 8; and Table 3 of Exhibit B, in bold), and that SEC1/FGF levels decline in MH7A cells (a synoviocyte cell line) following treatment with IL1β and TNFα ((See Patturajan Declaration, ¶ 9; and Table 4 of Exhibit B, in bold). These results demonstrate that SEQ ID 2 has utility as a probe for psoriasis and other inflammatory disorders (such as Crohn's Disease), and that SEQ ID NO: 1 or 2 may be a target for therapeutic agents in such cancers, as was disclosed in the as-filed specification at page 80, lines 20-22.

In the Office action, the Examiner references Skolnick *et al.* (Trends Biotechnol. 2000, 18:34-39) and Bork (Genome Res. 2000, 10:398-400) in support of the notion that function cannot be predicted based solely on structural similarity. However, Skolnick *et al.* in page 36, col. 2, line 2, clearly state: "For proteins whose sequence identity is above ~30%, one can use homology modeling to build the structure." At the time of filing this application Applicants' proteins of the invention possessed over 50% identity to the ADAMTS family of proteins. Furthermore, Bork indicates that protein function is context dependent, and both molecular and cellular aspects have to be considered (*See* Bork, page 398, col. 2, first paragraph, last line). Bork also acknowledges that sequence analysis is extremely powerful and that the generation of hypotheses derived by computational methods will be more and more often the first successful step in the design of experiments (page 400, column 2, penultimate paragraph, line 8).

Consistent with the teachings of the specification, the utilities known by those of ordinary skill in the art, and the data presented in the attached Exhibits, applicants respectfully submit that it is clear that the nucleic acids and polypeptides of the present invention can be used as probes for inflammatory and cell proliferative disorders, and as targets for therapeutic agents in such cancers, i.e., credible, specific and substantial utilities. Applicants assert that the functional attributes of the FGF family members are the functional attributes asserted by Applicants for SEQ ID NO: 2 in the specification. Accordingly, applicants submit that the claimed invention has a utility that is credible, substantial, and specific to the FGF family of proteins and therefore request withdrawal of the rejection under 35 U.S.C. §101.

The 35 USC § 112, first paragraph rejections

Claims 1-13 have been rejected under 35 U.S.C. § 112, first paragraph because one skilled in the art would not know how to use the invention since the claimed invention lacks utility. Applicants have overcome the Office's rejection under § 101 to the claims as amended above. Accordingly, the rejection under § 112, ¶ 1 should be withdrawn.

Claims 1-13 have also been rejected under 35 U.S.C. § 112, first paragraph because the subject matter described in the specification did not reasonably convey to one skilled in the art that the Applicant had possession of the claimed in invention at the time the application was filed. Claim 9 has been canceled herein. This objection is therefore moot in regard to this claim. The Examiner has rejected Claim 1 for referring to "90% identity." Claim 1 has been amended herein to delete this phrase. In regard to claims 3, 6 and 12, which recite "mutant or a variant," this phrase has been deleted from the pending claims 3, 6 and 12. Regarding claim 8, Applicants have amended this claim herein to delete the term "a portion" from the claim. Claim 1 (b) reciting "90% identical" has been canceled without any prejudice. Claims 3, 6 and 12 as amended no longer recite "mutant or variant". Claims 8 and 9 have been amended that they no longer recite "a portion". Accordingly, applicants believe that the rejections have now been rendered moot and request that such rejections be withdrawn.

The 35 USC § 112, Second paragraph rejections

Claims 5 and 6 have also been rejected under 35 U.S.C. § 112, second paragraph as indefinite for use of the phrase "an amino acid sequence of SEQ ID NO: 2." Applicants have amended claims 5 and 6 to delet the phrase "an amino acid sequence." This rejection can be withdrawn.

Claim 6 has also been rejected as indefinite for recitation of hybridization "under stringent conditions." Claim 6 does not recite this term. Claims 7 and 8 recite this term, and the further limitation "wherein said stringent conditions comprise hybridization in a high salt buffer comprising 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 mg/ml denatured salmon sperm DNA at 65°C." This rejection has been overcome and can be withdrawn.

The 35 USC § 102 rejections

Claims 1, 2, 8 and 9 have been rejected under 35 USC § 102(b) as being anticipated by Hopp et al., US Patent 5,011, 912. Claim 9 has been canceled herein. This objection is therefore moot in regard to this claim. Claim 1 has been amended herein to delete the phrase "90% identical." Claim 8 has been amended herein to delete the term "portion." In view of the amendments to claims 1 and 8, the pending claims are not anticipated by Hopp and the rejection should be withdrawn.

Claims 1-6 are also rejected under 35 USC 102 (a) as being anticipated by Kabnick et al., International Publication Number WO01/18228, published on March 15, 2001. The Examiner states that "[b]ecause the instant application does not meet the requirements of 35 USC § 112, first paragraph for the reasons given above, the priority to the earlier provisional application is denied." (See Office action, pages 13-14).

Applicants traverse the Examiner's denial, and the assignment of October 31, 2001 as the effective filing date of the instant application. As discussed *supra*, Applicants assert that the instant application meets the requirement of 35 USC §§ 101 and 112, first paragraph. Therefore, the instant application is entitled to the benefit of the earlier filing date under 35 USC § 120.

The instant application USSN 09/998,966 (Cura 51 CON-S2) claims priority to USSN 09/569,269 (Cura 51) filed May 11, 2000, which is pending, which in turn claims benefit of USSN 60/188,274 (Cura 51B) filed March 10, 2000, and USSN 60/134,315 (Cura 51) filed May 14, 1999, both of which are now abandoned. The earliest priority document USSN 60/134,315 (Cura 51) filed May 14, 1999, provided the amino acid sequence of SEQ ID NO: 2 (page 1, accession no: FGF 10 AC004449). Furthermore, USSN 60/188,274 (Cura 51B) describes cloning of SEQ ID NO: 2 (page 21-25, accession no: FGF 10 AC004449). A clustal alignment comparing the polypeptides SEQ ID NO:2 of the instant specification with that of USSN 60/134,315 and USSN 60/188,274 is provided as Appendix C for the Examiner's convenience.

Therefore, the instant application should be afforded the priority date of May 14, 1999, when determining patentability of the claims 1-6 over the cited WO 01/18228, in which case the rejection under 35 USC § 102(a) is inappropriate and should be withdrawn.

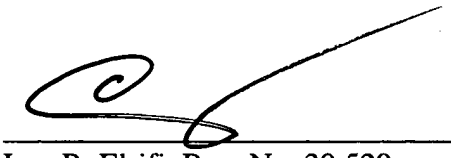
Double patenting

The Examiner has indicated that should claim 7 be found allowable, claim 8 will be objected to under 37 CFR § 1.75 for obviousness-type double patenting. In response, Applicants note that claim 7, as amended herein, recites "[a]n isolated nucleic acid hybridizes under stringent conditions with the nucleic acid of claim 1, wherein said stringent conditions comprise hybridization in a high salt buffer comprising 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 mg/ml denatured salmon sperm DNA at 65°C," while amended claim 8 recites "[a]n isolated nucleic acid that hybridizes under stringent conditions with the nucleic acid of SEQ ID NO: 1, wherein said stringent conditions comprise hybridization in a high salt buffer comprising 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 mg/ml denatured salmon sperm DNA at 65°C." As such, amended claim 8 is not a substantial duplicate of claim 7.

CONCLUSION

On the basis of the foregoing amendments and remarks, Applicants respectfully submit that this paper is fully responsive and that the pending claims are in condition for allowance. Such action is respectfully requested. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,



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Dated: August 8, 2003

Express Mail No.: US328702687US
Date of Deposit: August 8, 2003

Attorney Docket No.: 15966-551 CON-S2 (Cura-551 CON-S2)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS :	Richard A. Shimkets		
SERIAL NUMBER :	09/998,966	EXAMINER :	Olga N. Chernyshev
FILING DATE :	October 31, 2001	ART UNIT :	1646
FOR :	NOVEL NUCLEIC ACID SEQUENCES ENCODING HUMAN FIBROBLAST GROWTH FACTOR-LIKE POLYPEPTIDES		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

I, Meera Patturajan, hereby declare and state as follows:

1. I am employed by CuraGen, Inc., the assignee of this application. My title is research scientist. I received a Ph.D. in Microbiology and Cell Biology from Indian Institute of Science, India. I was a post-doctoral fellow at the Johns Hopkins University in Baltimore, Maryland from 1995 to 2000. I have been with CuraGen from 2000 to present.

2. I have read, and am familiar with, the contents of the United States patent application entitled "Novel Nucleic Acid Sequences Encoding Human Fibroblast Growth Factor-Like Polypeptides", serial number 09/998,966 which was filed October 31, 2001. I understand that the pending claims are directed to a nucleic acid encoding a polypeptide SEC1, SEQ ID NO: 22.

3. I am aware that the Examiner has issued an Office Action. In particular, I understand that the Examiner has rejected the pending claims under 35 U.S.C. §§ 101 and 112, contending that the pending claims are not supported by either a specific and substantial asserted utility or a well-established utility.

4. I make this declaration to rebut the Examiner's assertion, with which I do not agree. It is my opinion that the claimed compositions have a specific and substantial utility for at least the following reasons.

5. I have performed, or have had performed under my supervision, studies evaluating the recombinant expression of the SEC1/FGF (CG54455-06) which is a mature form of polypeptide of SEQ ID NO: 2 in mammalian CHO-K cells and bacterial E. coli cells. As shown in Figures 1a and 1b of Appendix A, a fusion protein comprising the CG54455-06 polypeptide migrates at approximately 88kD when produced in mammalian CHO-K cells, and approximately 24kD in E. coli cells, which is consistent with the highly modified nature of FGF polypeptides expressed in mammals. in tissue culture cells and in isolated normal and pathological human tissue. The methods used to perform these studies are described in the Appendix A.

6. I have also performed, or have had performed under my supervision, studies evaluating the proliferative effect of the SEC1/FGF (CG54455-06) polypeptide of SEQ ID NO: 2 in BaF3 cells recombinantly expressing the FGF receptor b2. As shown in Figure 2 of Appendix A, a CG54455-06 fusion protein-enriched conditioned media comprising the CG54455-06 polypeptide induces DNA synthesis in BaF3R2b cells as measured by BrdU incorporation. As shown in Figures 3 and 4 of Appendix A, a recombinantly expressed polypeptide comprising a mature form of the SEC1/FGF polypeptide (Figure 3 of Appendix A) and a fusion protein comprising the CG54455-06 polypeptide (Figure 4 of Appendix A) induce DNA synthesis in BaF3R2b cells in a dose-dependent manner, as measured by BrdU incorporation. We also demonstrate that the stimulatory effect of the SEC1/FGF polypeptide or a fusion protein comprising the CG54455-06 polypeptide is specific for cells expressing the FGFR2b, as no stimulatory effect of these polypeptides was detected in BaF3 cells expressing other FGF receptors, such as FGFR1c, FGFR2c, FGFR3b, or FGFR3c (See Figure 5 of Appendix A).

7. I have further performed, or have had performed under my supervision, studies evaluating the quantitative expression and sequence homology of the nucleic acid of SEQ ID NO: 1 encoding the polypeptide of SEQ ID NO: 2 (also known as CG54455-06) in tissue culture cells and in isolated normal and pathological human tissue. The methods used to perform these studies are described in the Appendix B attached hereto.

8. In a first study, results provided in Table 3 in Appendix B using specific probe/primer set (Ag4347, which are shown in Table 2), show that expression of the SEC1/FGF, CG54455-06 gene is downregulated in Psoriasis patients and is upregulated in its corresponding matched controls. Thus, gene or proteins levels of expression are useful as a diagnostic marker for psoriasis. This will also have important implications in wound healing and other inflammatory conditions. Furthermore, therapeutic modulation of the expression or function of this gene is useful in the treatment of inflammatory disorders.

7. In a second study, results using Ag4346 probe/primer sets, shown in Table 1 in Appendix B, show decreased expression of the CG54455-06 gene in a cluster of samples derived from MH7A synoviocyte cells treated with anti-inflammatory compounds such as IL1b and TNFα. Thus, gene or proteins levels of expression are useful to differentiate between these samples and other samples on this panel and as a marker to detect the presence of inflammatory disorders. Furthermore, gene, protein, antibodies or small molecule therapeutics targeting this gene or its protein product are effective in the treatment of inflammatory disorders.

8. The results of these studies, in my opinion, demonstrate that the polypeptide can be used in therapeutic and diagnostic applications in inflammatory disorders. Thus, I believe that the Examiner should withdraw the rejection and allow the pending claims.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, Title 18, United States Code, and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.

Meera Patturajan

Signed at Branford CT
this 8th day of August, 2003

Express Mail No.: US328702687US
Date of Deposit: August 8, 2003

APPENDIX A

Expression of CG54455-06 in stable CHO-K1 cells

A 456 bp long BglII-XhoI fragment containing the CG54455-06 (mature form of CG54455-01) sequence was subcloned into BamHI-XhoI digested pEE14.4FL2_MSA to generate plasmid 3337. The resulting plasmid 3337 was transfected into CHO-K1 cells using the LipofectaminePlus reagent following the manufacturer's instructions (Invitrogen/Gibco) and stable clones were selected based on resistance against MSX. The culture media was DMEM, 10% FBS, 1x nonessential amino acids. The expression and secretion levels of the clone were assessed by Western blot analysis using HRP conjugated V5 antibody. The V5 epitope is fused to the gene of interest at the Cter, in the pEE14.4Sec vector. Figure 1a shows that CG54455 is expressed, and a 88 kDa protein is secreted by the CHO-K1 cells.

Purification of CG54455-06 expressed in stable CHO-K1 cells

CG54455-MSA fusion protein enriched conditioned medium (10 L) generated by CHO-K stable transfectants (figure 1) was clarified by filtration, and passed through a metal chelation column (50 mL Pharmacia column). The protein was eluted by step elution using buffer containing 25mM, 50mM, 100mM, and 500mM imidazole. The eluted protein was further subjected to intermediate purification on a 5 ml metal chelation column (Pharmacia) and eluted with a linear gradient from 0 to 500mM imidazole. The final protein fraction was dialyzed against 20mM Tris-HCl, pH7.4 + 150mM NaCl. Protein samples were stored at –70°C.

Expression and Purification of CG54455-06 in *E. coli* strain BL21(DE3)

A 456 bp long BglII-XhoI fragment containing the CG54455-06 (mature form of CG54455-01) was subcloned into BamHI-XhoI digested pETMY-His (Invitrogen) to generate plasmid 2021. The resulting plasmid 2021 was transformed into *E. coli* using the standard transformation protocol. The cells were harvested 2 h post induction with IPTG and disrupted by sonication. The sonicate was brought to a final concentration of 0.5 M NaCl and was passed through a metal chelation column (5 ml Amersham HiTrap metal chelate column). The final protein fraction was eluted using 1X phosphate buffered saline (Mediatech Cellgro, VA) containing 0.4 M NaCl and 500mM imidazole. Protein samples were stored at 4°C. The expression and purification of CG54455-06 were assessed by Western blot analysis using HRP conjugated anti-His antibody. CG54455-06 was expressed as a 24 kDa protein in *E.coli* (Figure 1b).

Figure1a. CG54455-MSA fusion protein secreted by CHO-K cells.

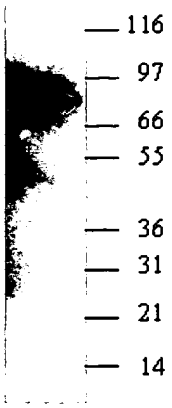
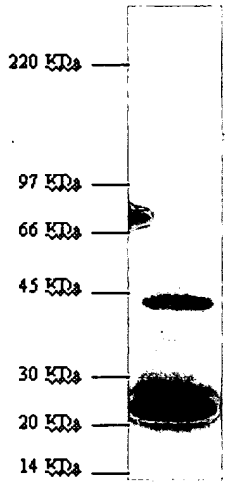


Figure 1b. CG54455-06 protein expressed by *E.coli* strain BL21.



Cellular Proliferation Responses and Receptor Specificity with CG54455

Novel members of the FGF family could have significant therapeutic potential in diseases associated with cell and tissue remodeling, as these growth factors regulate diverse cellular functions such as growth, survival, apoptosis, motility and differentiation (Szebenyi G and Fallon JF, Int Rev Cytol 1999 185:45-106). BrdU incorporation (proliferation assay) was performed to characterize the biological activity of the novel human FGF-22, CG54455. Fibroblast growth factors are known to have both stimulatory and inhibitory effects on wide variety of cell types. The proliferative response of BaF3R2b (BaF3 cells expressing the FGFR 2b receptor) to mature CG54455-06 and CG54455-06 fused to MSA were also evaluated. Baf3 cells that stably express various alpha isoforms of the fibroblast growth factor receptors (FGFRs) (Ornitz et al., 1996, Receptor specificity of the Fibroblast growth factor family. J. Biol. Chem., 271 (25):15292-15297) were also used for evaluation.

BrdU Incorporation. Proliferative activity was measured by treatment of cultured BaF3 R2b cells with either conditioned media containing CG54455-fusion protein, or purified CG54455-06 protein for 72 h, followed by measurement of BrDU incorporation during DNA synthesis. BaF3R2b were cultured in RPMI supplemented with 5 % fetal bovine serum, beta mercaptoethanol (55 uM), and 5 ug/ml heparin. Cells were incubated with BrdU (10 μM final concentration) for 3 h and BrdU incorporation was assayed according to the manufacturer's specifications (Boehringer Mannheim, Indianapolis, IN).

Proliferation of Baf3R2b cells by conditioned media containing CG54455-MSA fusion protein. CG54455-MSA fusion protein enriched conditioned medium (CM-FGF-22) were generated by incubating the CHO-K transfectants in medium containing 5 %FBS for 32h (described in example 3, figure 1a). Non-transfected CHO-K cells were used to generate

conditioned medium control (CM-CT). Baf3R2b cells were treated with CG54455-MSA enriched conditioned medium and the control media at different dilution as shown in the figure 2. The results showed that conditioned medium from MSA-FGF-22 CHO-K1 transfectants demonstrated a proliferative activity on BaF3 R2b cells (Figure. 2). The positive control, FGF-10 also showed a proliferative activity on BaF3R2b.

Proliferation of Baf3R2b cells by CG54455-06 and CG54455-MSA fusion protein. Baf3R2b cells were treated with purified CG54455-06 mature protein (FGF22), CG54455-MSA fusion protein (FGF-MSA), control MSA protein and control FGF10 (KGF2) at different concentration as shown in the Figure 3 and 4 respectively. CG54455-06, and CG54455-MSA was found to induce DNA synthesis in the Baf3R2b cell line in a dose-dependent manner.

Figure 2.

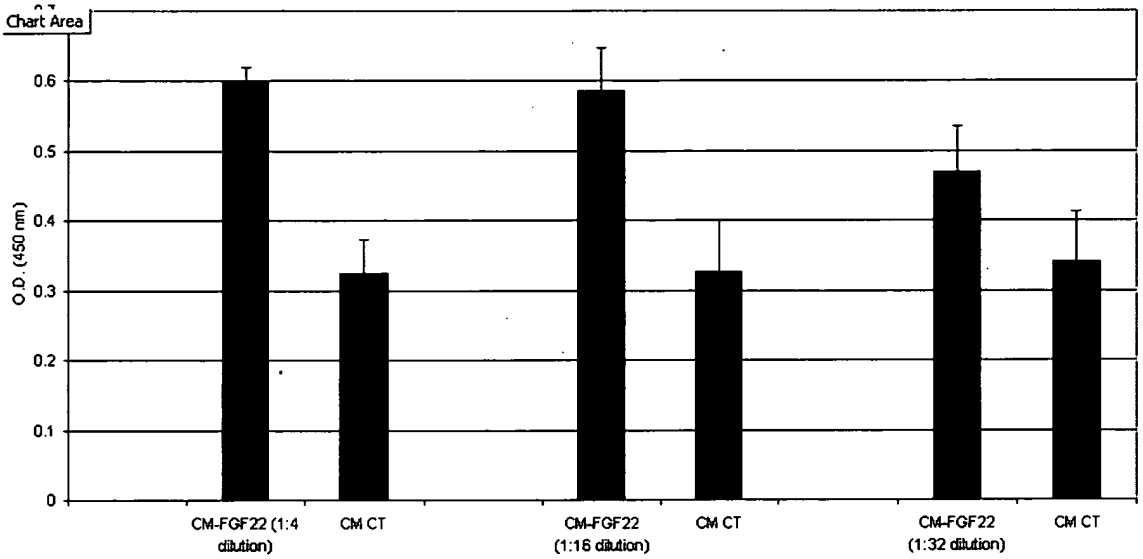


Chart Area
O.D. (450 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
CM-FGF22 (1:4 dilution)
CM CT
CM-FGF22 (1:16 dilution)
CM CT
CM-FGF22 (1:32 dilution)
CM CT

Figure. 3.

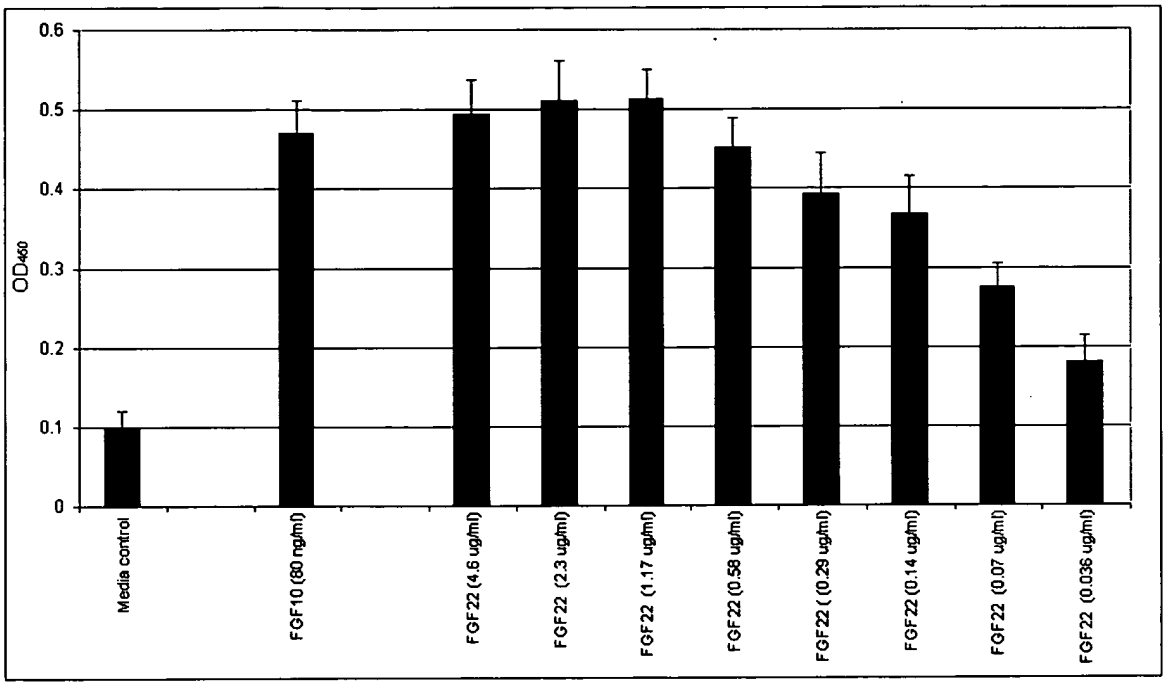
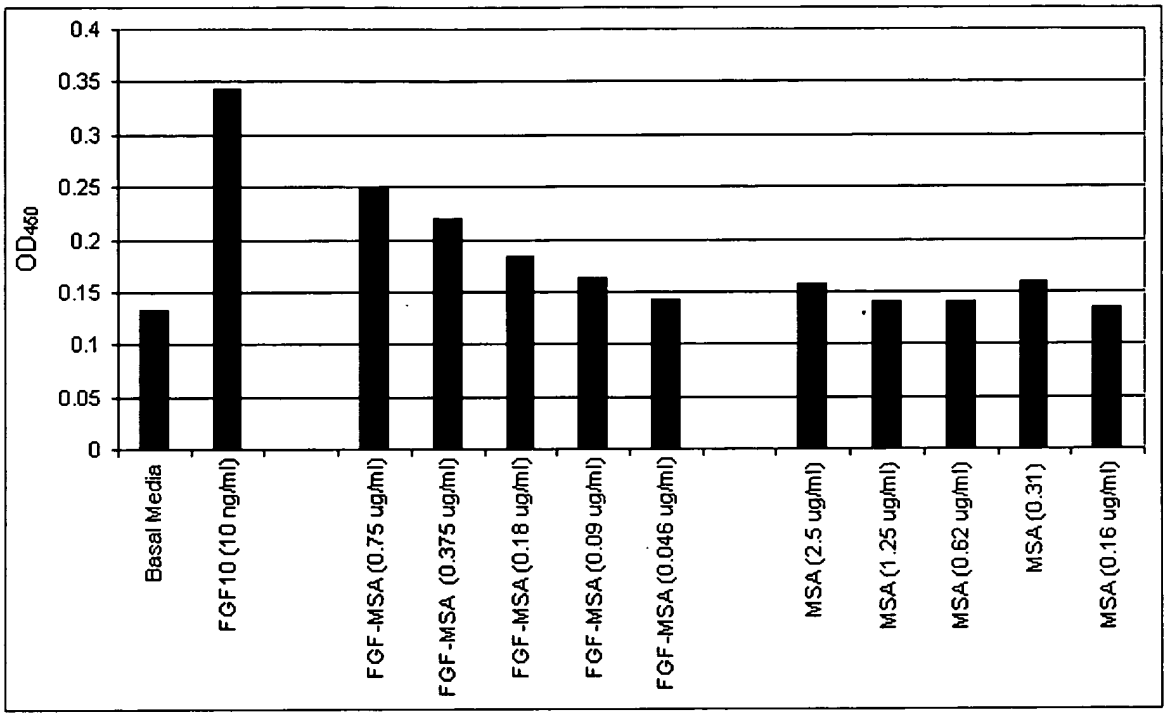
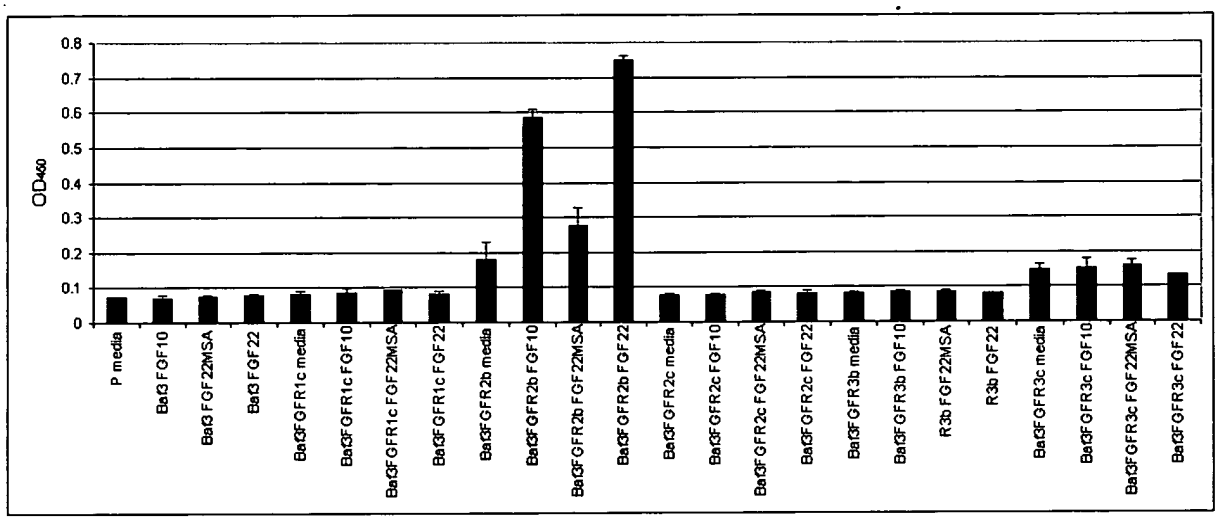

0.6
0.5
0.4
0.3
0.2
0.1
0
OD450
Media control
FGF10 (80 ng/ml)
FGF22 (4.6 ug/ml)
FGF22 (2.3 ug/ml)
FGF22 (1.17 ug/ml)
FGF22 (0.58 ug/ml)
FGF22 ((0.29 ug/ml)
FGF22 (0.14 ug/ml)
FGF22 (0.07 ug/ml)
FGF22 (0.036 ug/ml)

Figure. 4.


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
OD450
Basal Media
FGF10 (10 ng/ml)
FGF-MSA (0.75 ug/ml)
FGF-MSA (0.375 ug/ml)
FGF-MSA (0.18 ug/ml)
FGF-MSA (0.09 ug/ml)
FGF-MSA (0.046 ug/ml)
MSA (2.5 ug/ml)
MSA (1.25 ug/ml)
MSA (0.62 ug/ml)
MSA (0.31)
MSA (0.16 ug/ml)

Receptor specificity of CG54455-06 and CG54455-MSA fusion protein. Baf3 cells expressing the different FGFRs (Ornitz et al., 1996, Receptor specificity of the Fibroblast growth facotor family. J. Biol. Chem., 271 (25):15292-15297) were treated with CG54455-06 mature protein (FGF22, 1.5 μg/ml), CG54455-MSA fusion protein (FGF-MSA, 0.75 μg/ml), and control FGF10 (KGF2 20 ng/ml). As shown in the Figure 5, FGF10, CG54455-06, and CG54455-MSA fusion protein were found to induce DNA synthesis specifically in the Baf3R2b cell line (specific for FGFR2b).

Figure. 5.



Appendix B

Quantitative expression analysis of clones in various cells and tissues

The quantitative expression of various NOV genes was assessed using microtiter plates containing RNA samples from a variety of normal and pathology-derived cells, cell lines and tissues using real time quantitative PCR (RTQ-PCR) performed on an Applied Biosystems (Foster City, CA) ABI PRISM® 7700 or an ABI PRISM® 7900 HT Sequence Detection System.

RNA integrity of all samples was determined by visual assessment of agarose gel electropherograms using 28S and 18S ribosomal RNA staining intensity ratio as a guide (2:1 to 2.5:1 28s:18s) and the absence of low molecular weight RNAs (degradation products). Control samples to detect genomic DNA contamination included RTQ-PCR reactions run in the absence of reverse transcriptase using probe and primer sets designed to amplify across the span of a single exon.

RNA samples were normalized in reference to nucleic acids encoding constitutively expressed genes (i.e., β-actin and GAPDH). Alternatively, non-normalized RNA samples were converted to single strand cDNA (sscDNA) using Superscript II (Invitrogen Corporation, Carlsbad, CA, Catalog No. 18064-147) and random hexamers according to the manufacturer's instructions. Reactions containing up to 10 μg of total RNA in a volume of 20 μl or were scaled up to contain 50 μg of total RNA in a volume of 100 μl and were incubated for 60 minutes at 42°C. sscDNA samples were then normalized in reference to nucleic acids as described above.

Probes and primers were designed according to Applied Biosystems *Primer Express* Software package (version I for Apple Computer's Macintosh Power PC) or a similar algorithm using the target sequence as input. Default reaction condition settings and the following parameters were set before selecting primers: 250 nM primer concentration; 58°-60° C primer melting temperature (Tm) range; 59° C primer optimal Tm; 2° C maximum primer difference (if probe does not have 5' G, probe Tm must be 10° C greater than primer Tm; and 75 bp to 100 bp amplicon size. The selected probes and primers were synthesized by Synthegen (Houston, TX). Probes were double purified by HPLC to remove uncoupled dye and evaluated by mass spectroscopy to verify coupling of reporter and quencher dyes to the 5' and 3' ends of the probe, respectively. Their final concentrations were: 900 nM forward and reverse primers, and 200nM probe.

Normalized RNA was spotted in individual wells of a 96 or 384-well PCR plate (Applied Biosystems, Foster City, CA). PCR cocktails included a single gene-specific probe and primers set or two multiplexed probe and primers sets. PCR reactions were done using TaqMan® One-Step RT-PCR Master Mix (Applied Biosystems, Catalog No. 4313803) following manufacturer's instructions. Reverse transcription was performed at 48° C for 30 minutes followed by amplification/PCR cycles: 95° C 10 min, then 40 cycles at 95° C for 15 seconds, followed by 60° C for 1 minute. Results were recorded as CT values (cycle at which a given sample crosses a threshold level of fluorescence) and plotted using a log scale, with the difference in RNA concentration between a given sample and the sample with the lowest CT value being represented as 2 to the power of delta CT. The percent relative expression was the reciprocal of the RNA difference multiplied by 100. CT values below 28 indicate high expression, between 28

and 32 indicate moderate expression, between 32 and 35 indicate low expression and above 35 reflect levels of expression that were too low to be measured reliably.

Normalized sscDNA was analyzed by RTQ-PCR using 1X TaqMan® Universal Master mix (Applied Biosystems; catalog No. 4324020), following the manufacturer's instructions. PCR amplification and analysis were done as described above.

AI_comprehensive panel_v1.0

Autoimmunity (AI) comprehensive panel v1.0 included two controls and 89 cDNA test samples isolated from male (M) and female (F) surgical and postmortem human tissues that were obtained from the Backus Hospital and Clinomics (Frederick, MD). Tissue samples included : normal, adjacent (Adj); matched normal adjacent (match control); joint tissues (synovial (Syn) fluid, synovium, bone and cartilage, osteoarthritis (OA), rheumatoid arthritis (RA)); psoriatic; ulcerative colitis colon; Crohns disease colon; and emphysmatic, asthmatic, allergic and chronic obstructive pulmonary disease (COPD) lung.

Cellular OA/RA panel

Cellular OA.RA panel includes 2 control wells and 35 test samples comprised of cDNA generated from total RNA isolated from human cell lines or primary cells representative of the human joint and its inflammatory condition. Cell types included normal human osteoblasts (Nhost) from Clonetics (Cambrex, East Rutherford, NJ), human chondrosarcoma SW1353 cells from ATCC (Manossas, VA)), human fibroblast-like synoviocytes from Cell Applications, Inc. (San Diego, CA) and MH7A cell line (a rheumatoid fibroblast-like synoviocytes transformed with SV40 T antigen) from Riken Cell bank (Tsukuba Science City, Japan). These cell types were activated by incubating with various cytokines (IL-1 beta ~1-10 ng/ml, TNF alpha ~5-50 ng/ml, or prostaglandin E2 for Nhost cells) for 1. 6, 18 or 24 h. All these cells were starved for at least 5 h and cultured in their corresponding basal medium with ~ 0.1 to 1 % FBS.

A. CG54455-01: (also known as FGF10AC004449)

Expression of gene CG54455-01 was assessed using the primer-probe sets Ag4346, and Ag4347, described in Tables 1 and 2. Results of the RTQ-PCR runs are shown in Tables 3, 4, 5, 6 and 7.

Table 1. Probe Name Ag4346

Primers	Sequences	Length	Start Position	SEQ ID No
Forward	5'-cgtggtcatcaaagcagtgt-3'	20	378	
Probe	TET-5'-ctcaggcttctacgtggccatgaac-3'-TAMRA	25	399	
Reverse	5'-tgcagtccacggtgtagagt-3'	20	450	

Table 2. Probe Name Ag4347

Primers	Sequences	Length	Start	SEQ ID

			Position	No
Forward	5'-tggagatccgctctgtacacg-3'	21	350	
Probe	TET-5'-cctgaggacactgctttgatgaccacg-3'-TAMRA	27	378	
Reverse	5'-cggttcatggccacgtaga-3'	19	407	

Table 3. AI_comprehensive panel_v1.0

Column A - Rel. Exp.(%) Ag4347, Run 278182082			
Tissue Name	**A**	**Tissue Name**	**A**
110967 COPD-F	10.0	112427 Match Control Psoriasis-F	60.3
110980 COPD-F	19.2	112418 Psoriasis-M	12.1
110968 COPD-M	13.3	112723 Match Control Psoriasis-M	0.8
110977 COPD-M	59.5	112419 Psoriasis-M	4.9
110989 Emphysema-F	16.4	112424 Match Control Psoriasis-M	15.7
110992 Emphysema-F	14.3	112420 Psoriasis-M	15.2
110993 Emphysema-F	6.2	112425 Match Control Psoriasis-M	30.1
110994 Emphysema-F	4.1	104689 (MF) OA Bone-Backus	21.5
110995 Emphysema-F	19.3	104690 (MF) Adj "Normal" Bone-Backus	10.9
110996 Emphysema-F	9.3	104691 (MF) OA Synovium-Backus	11.4
110997 Asthma-M	1.7	104692 (BA) OA Cartilage-Backus	27.4
111001 Asthma-F	4.5	104694 (BA) OA Bone-Backus	5.4
111002 Asthma-F	15.7	104695 (BA) Adj "Normal" Bone-Backus	36.1
111003 Atopic Asthma-F	14.0	104696 (BA) OA Synovium-Backus	2.0
111004 Atopic Asthma-F	21.5	104700 (SS) OA Bone-Backus	4.7
111005 Atopic Asthma-F	6.4	104701 (SS) Adj "Normal" Bone-Backus	15.0
111006 Atopic Asthma-F	5.1	104702 (SS) OA Synovium-Backus	10.6
111417 Allergy-M	18.7	117093 OA Cartilage Rep7	7.2
112347 Allergy-M	4.8	112672 OA Bone5	2.5
112349 Normal Lung-F	2.7	112673 OA Synovium5	3.4
112357 Normal Lung-F	**100.0**	112674 OA Synovial Fluid cells5	9.9
112354 Normal Lung-M	8.3	117100 OA Cartilage Rep14	2.7
112374 Crohns-F	19.9	112756 OA Bone9	18.7
112389 Match Control Crohns-F	33.9	112757 OA Synovium9	4.3
112375 Crohns-F	6.7	112758 OA Synovial Fluid Cells9	4.7
112732 Match Control Crohns-F	31.4	117125 RA Cartilage Rep2	3.1
112725 Crohns-M	9.1	113492 Bone2 RA	5.8
112387 Match Control Crohns-M	2.2	113493 Synovium2 RA	1.8
112378 Crohns-M	4.3	113494 Syn Fluid Cells RA	8.0
112390 Match Control Crohns-M	28.7	113499 Cartilage4 RA	2.6

112726 Crohns-M	1.4	113500 Bone4 RA	10.4
112731 Match Control Crohns-M	15.3	113501 Synovium4 RA	5.6
112380 Ulcer Col-F	4.4	113502 Syn Fluid Cells4 RA	5.3
112734 Match Control Ulcer Col-F	18.8	113495 Cartilage3 RA	5.9
112384 Ulcer Col-F	7.0	113496 Bone3 RA	3.5
112737 Match Control Ulcer Col-F	1.2	113497 Synovium3 RA	0.4
112386 Ulcer Col-F	19.6	113498 Syn Fluid Cells3 RA	1.6
112738 Match Control Ulcer Col-F	1.0	117106 Normal Cartilage Rep20	4.2
112381 Ulcer Col-M	0.0	113663 Bone3 Normal	0.0
112735 Match Control Ulcer Col-M	0.0	113664 Synovium3 Normal	0.0
112382 Ulcer Col-M	7.1	113665 Syn Fluid Cells3 Normal	3.6
112394 Match Control Ulcer Col-M	11.9	117107 Normal Cartilage Rep22	3.3
112383 Ulcer Col-M	8.4	113667 Bone4 Normal	10.7
112736 Match Control Ulcer Col-M	29.1	113668 Synovium4 Normal	16.4
112423 Psoriasis-F	1.0	113669 Syn Fluid Cells4 Normal	4.2

Table 4. Cellular OA/RA

Column A - Rel. Exp.(%) Ag4346, Run 406013342			
Tissue Name	**A**	**Tissue Name**	**A**
158667 Nhost medium 1h	8.5	164336 SW1353 + TNF-a (100 ng/ml) 6h	24.5
158670 Nhost + IL-1b (10 ng/ml), 1h	3.5	164337 SW1353 medium alone 18h	49.7
158673 Nhost + PGE2 (10-6M) 1h	18.2	164338 SW1353 + IL-1b (1 ng/ml) 18h	38.2
158668 Nhost medium alone 6h	26.6	164339 SW1353 + IL-1b (10 ng/ml) 18h	34.9
158671 Nhost + IL-1b (10 ng/ml) 6h	29.3	164340 SW1353 + TNF-a (10ng/ml) 18h	19.9
158674 Nhost + PGE2 (10-6M) 6 h	2.3	164341 SW1353 + IL-1b (100 ng/ml) 18h	55.1
158669 Nhost medium alone 24 h	0.0	173326 HFLS-RA (cell aplication) medium alone 18 h	12.2
158672 Nhost + IL-1b (10 ng/ml) 24h	33.7	173327 HFLS-RA (cell aplication) + TNF-a 18h	36.9
158675 Nhost + PGE2 (10-6M) 24 h	41.5	173331 MH7A (synoviocyte cell line) medium 1h	36.6
164327 SW1353 medium alone 1h	66.0	173332 MH7A (synoviocyte cell line) + IL1b 1h	15.3
164328 SW1353 + IL-1b (1 ng/ml) 1h	22.5	173334 MH7A (synoviocyte cell line) TNFa 1h	0.0
164329 SW1353 + IL-1b (10 ng/ml) 1h	26.8	173336 MH7A (synoviocyte cell line) medium alone 6h	**100.0**
164330 SW1353 +TNF-a (10 ng/ml) 1h	20.4	173339 MH7A (synoviocyte cell line) + IL1b 6h	77.9

164331 SW1353 +TNF-a (100 ng/ml) 1h	51.1	173341 MH7A (synoviocyte cell line) TNFa 6h	7.6
164332 SW1353 medium alone 6h	42.6	173342 MH7A (synoviocyte cell line) medium alone 18h	15.1
164333 SW1353 + IL-1b (1 ng/ml) 6h	41.5	173344 MH7A (synoviocyte cell line) + IL1b 18h	30.8
164334 SW1353 + IL-1b (10 ng/ml) 6h	88.3	173346 MH7A (synoviocyte cell line) TNF-a 18h	25.2
164335 SW1353 + TNF-a (10 ng/ml) 6h	34.4		

AI_comprehensive panel_v1.0 Summary: Ag4347 Highest expression of this gene was detected in normal lung (CT=30.8). This gene showed a wide spread low expression in this panel. Moderate to low levels of expression of this gene were detected in samples derived from normal and osteoarthritis/rheumatoid arthritis bone, cartilage, and synovium samples, from normal lung, COPD lung, emphysema, atopic asthma, asthma, allergy, Crohn's disease (normal matched control and diseased), ulcerative colitis(normal matched control and diseased), and psoriasis (normal matched control and diseased). Therefore, therapeutic modulation of this gene, encoded protein and/or use of antibodies or small molecule targeting this gene or gene product will ameliorate symptoms/conditions associated with autoimmune and inflammatory disorders including psoriasis, allergy, asthma, inflammatory bowel disease, rheumatoid arthritis and osteoarthritis

Cellular OA/RA Summary: Ag4346 Highest expression of this gene is detected in MH7A (synoviocyte) cell line (CT=33.5). Low expression of this gene is also detected in untreated and activated SW1353 (chondrocyte) cell lines, and activated MH7A cells. Therefore, modulation of this gene or encoded protein will be useful in the treatment of orthoarthritis and rheumatoid arthritis.

Appendix C

ClustalW Protein Alignment Analysis

Sequences analyzed:

1. 60_188274
2. 60_134315
3. SEQID_2

Clustal Details:

```
CLUSTAL W (1.7) Multiple Sequence Alignments


Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: 60_188274       170 aa
Sequence 2: 60_134315       170 aa
Sequence 3: SEQID_2         170 aa
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  100
Sequences (1:3) Aligned. Score:  100
Sequences (2:3) Aligned. Score:  100
Start of Multiple Alignment
There are 2 groups
Aligning...
Group 1: Sequences:   2      Score:2380
Group 2: Sequences:   3      Score:2380
Alignment Score 3375
CLUSTAL-Alignment file created  [/opt/curagen/curatool/data/working/ksanth_183_clustalwp.align]
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Multiple Alignment:

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60_134315    1 MRRRLWLGLAWLLLARAPDAAGTPSASRGPRSYPHLEGDVRWRRLFSSTHFFLRVDPGGR  60
SEQID_2      1 MRRRLWLGLAWLLLARAPDAAGTPSASRGPRSYPHLEGDVRWRRLFSSTHFFLRVDPGGR  60

60_188274   61 VQGTRWRHGQDSILEIRSVHVGVVVIKAVSSGFYVAMNRRGRLYGSRLYTVDCRFRERIE  120
60_134315   61 VQGTRWRHGQDSILEIRSVHVGVVVIKAVSSGFYVAMNRRGRLYGSRLYTVDCRFRERIE  120
SEQID_2     61 VQGTRWRHGQDSILEIRSVHVGVVVIKAVSSGFYVAMNRRGRLYGSRLYTVDCRFRERIE  120

60_188274  121 ENGHNTYASQRWRRRGQPMFLALDRRGGPRPGGRTRRYHLSAHFLPVLVS  170
60_134315  121 ENGHNTYASQRWRRRGQPMFLALDRRGGPRPGGRTRRYHLSAHFLPVLVS  170
SEQID_2    121 ENGHNTYASQRWRRRGQPMFLALDRRGGPRPGGRTRRYHLSAHFLPVLVS  170
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Fibroblast growth factor 22 and its potential role during skin development and repair

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Abstract

We have isolated, using RT–PCR, a cDNA from mouse skin wounds that encodes fibroblast growth factor (FGF) 22, a recently discovered member of the FGF family, which is closely related to FGF-7 and FGF-10. Transient expression of tagged FGF-22 protein in COS-1 and MCF-7 cells revealed that the protein was present within the cell and at the cell surface but was not apparently released from the cell. Analysis of RNA expression revealed that FGF-22 transcripts were not detected in the developing mouse embryo until day E16.5 and in the adult mouse it was expressed in the brain, tongue, and skin, but not in other tissues examined. After skin injury, FGF-22 mRNA levels were slightly down-regulated within the first 5 days after wounding, but expression increased strongly at the later stages of the repair process. In situ hybridization revealed the presence of FGF-22 mRNA throughout the epidermis and hair follicle keratinocytes of E16.5 embryos, as well as in adult skin and keratinocytes of the hyperthickened wound epithelium. This expression pattern suggests a potential role for FGF-22 in cutaneous development and repair.

Keywords: Keratinocyte growth factor; Skin; Epidermis; Dermis; Wound healing

Introduction

The fibroblast growth factor (FGF) family of mitogens comprises at least 22 structurally related growth factors [reviewed in 1]. They are between 155 and 268 amino acids in length and share a conserved central region. They bind heparin, heparan sulfate proteoglycans, and glycosaminoglycans and strongly concentrate in the extracellular matrix [reviewed in 2].

Most members of the FGF family regulate proliferation, differentiation, migration, and/or survival of a wide variety of different target cells [reviewed in 1, 2]. The only known exception is FGF-7 (keratinocyte growth factor; KGF), which specifically acts on epithelial cells, at least in the adult mouse [3; reviewed in 4]. It is produced by various types of mesenchymal cells in vitro and in vivo, but not by epithelial cells. By contrast, most cells of epithelial origin express FGFR2-IIIb, the only known high-affinity receptor for FGF-7 [5]. These results suggested that FGF-7 acts predominantly in a paracrine manner. Such a paracrine action of FGF-7 seems to occur in normal and particularly in wounded skin. We and others have demonstrated a weak expression of FGF-7 in murine and human skin, but upon skin injury there is a striking induction of this growth factor in dermal fibroblasts [6,7]. By contrast, FGFR2-IIIb was exclusively expressed on keratinocytes of the epidermis and the hair follicles. This expression pattern of FGF-7 and its receptor suggested that dermally derived FGF-7 stimulates wound reepithelialization in a paracrine manner. This hypothesis was strongly supported by the wound-healing phenotype seen in transgenic mice that express a dominant-negative FGFR2-IIIb mutant in the basal keratinocytes of the epidermis and in the outer root sheath keratinocytes of the hair follicles. These mice were characterized by a severe delay in wound reepithelialization [8], demonstrating the importance of FGFR2-IIIb signaling during cutaneous wound repair. Surprisingly, mice lacking FGF-7 revealed no phenotypic abnormalities and even the healing process of incisional wounds appeared normal [9], suggesting that other FGFR2-IIIb ligands can compensate for

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E-mail address: richard.grose@cancer.org.uk (R. Grose).

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doi:10.1016/S0014-4827(03)00139-3

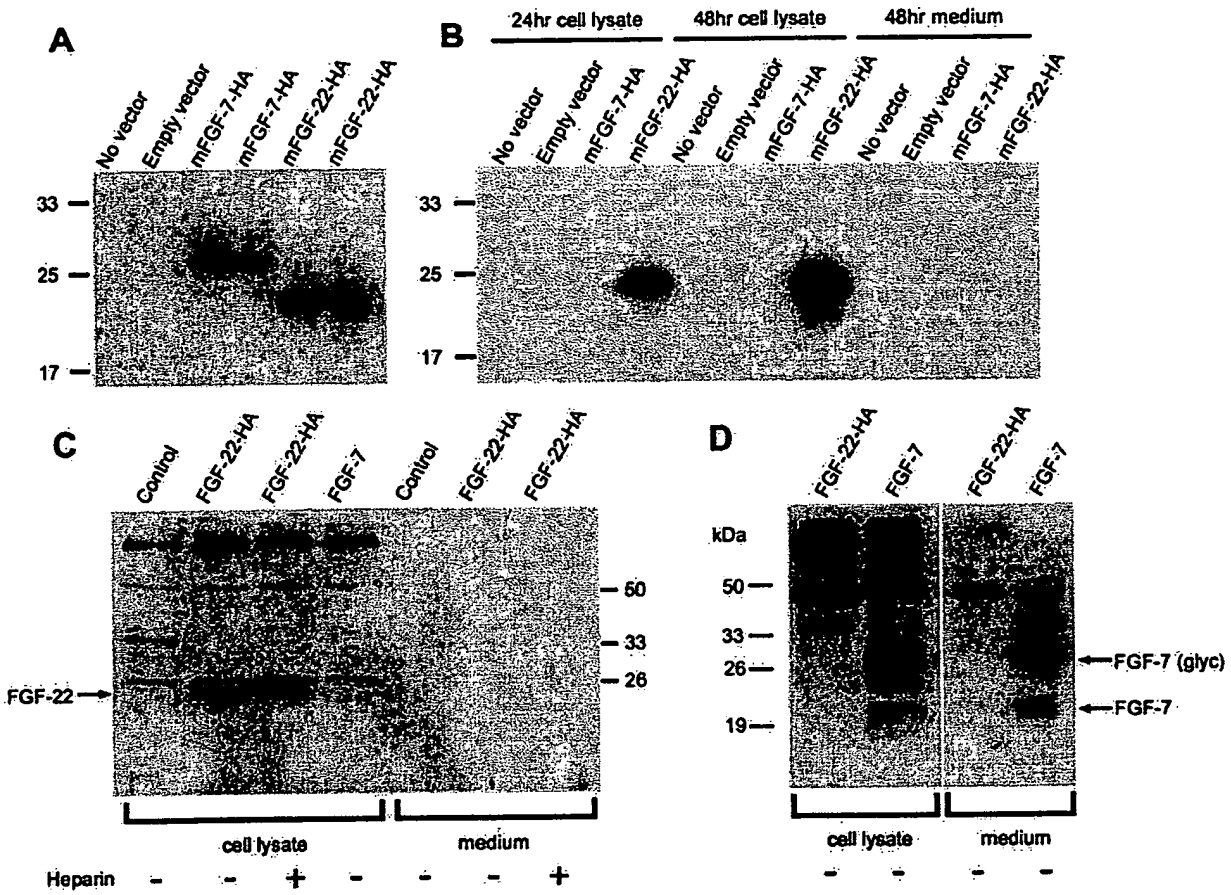


Fig. 1. FGF-22 is a cell-associated protein. (A) Cell lysates of transiently transfected COS-1 cells were analysed in duplicate for the presence of tagged FGF-7 and FGF-22 proteins using an antibody directed against the HA epitope. Controls were no vector and empty pCG vector. (B) COS-1 cells were transiently transfected (same constructs as in A) and cell lysates (24 and 48-h posttransfection) and conditioned medium (48-h posttransfection) were analysed by immunoblotting for the presence of FGF-22 protein using a polyclonal antiserum directed against a carboxyterminal peptide of this growth factor. A specific band was seen in the lysate of cells transfected with FGF-22, and the antibody showed no cross-reactivity with FGF-7 protein. (C) Total cell lysates and concentrated conditioned medium of transfected COS-1 cells were analysed for the presence of FGF-22 protein using an antibody directed against the HA epitope. The control lane represents cells transfected with a control vector containing an insert that encodes an unrelated cytoplasmic HA-tagged 35-kDa protein. The FGF-7 was nontagged. Cells were cultured in the presence or absence of 0.005 U/ml heparin, as indicated. (D) As a positive control for secretion, COS-1 cells were transfected with nontagged FGF-7 expression plasmid. Cell lysates and conditioned media of these cells were analysed by Western blotting using a FGF-7-specific antiserum that recognises the glycosylated (glyc) and nonglycosylated forms of FGF-7 [14], but not FGF-22. Since FGF-7 is efficiently secreted, the sample was diluted 1:3 with 1× Laemmli buffer prior to loading.

the lack of this growth factor. A potential candidate is FGF-10, another FGF family member that has high homology to FGF-7 and also binds to FGFR2-IIIb with high affinity [10,11]. Furthermore, an additional FGF-7/FGF-10 homologue was recently described and designated FGF-22 [12]. However, little is currently known about its expression pattern and the corresponding protein has as yet not been characterised.

Materials and methods

Cloning of murine FGF-22

The full-length murine FGF-22 cDNA was amplified by RT–PCR, using RNA from 10-day mouse wounds. The 5′ primer included the ATG and 15 additional nucleotides of the coding region. The 3′ primer included the last 18 nucleotides of the FGF-22 open reading frame. The stop codon was replaced by a restriction site that allowed the fusion of the open reading frame with an hemagglutinin (HA) epitope present in the eukaryotic pCG expression vector. In the latter, expression of cDNAs is driven by the CMV promoter. After insertion of the amplified fragment into this expression vector, it was fully sequenced and used for transient transfections.

Transfection of COS-1 cells, MCF-7 cells and primary human keratinocytes

COS-1 and MCF-7 cells were cultured in DMEM containing 10% FCS. For transient transfection, Lipo-

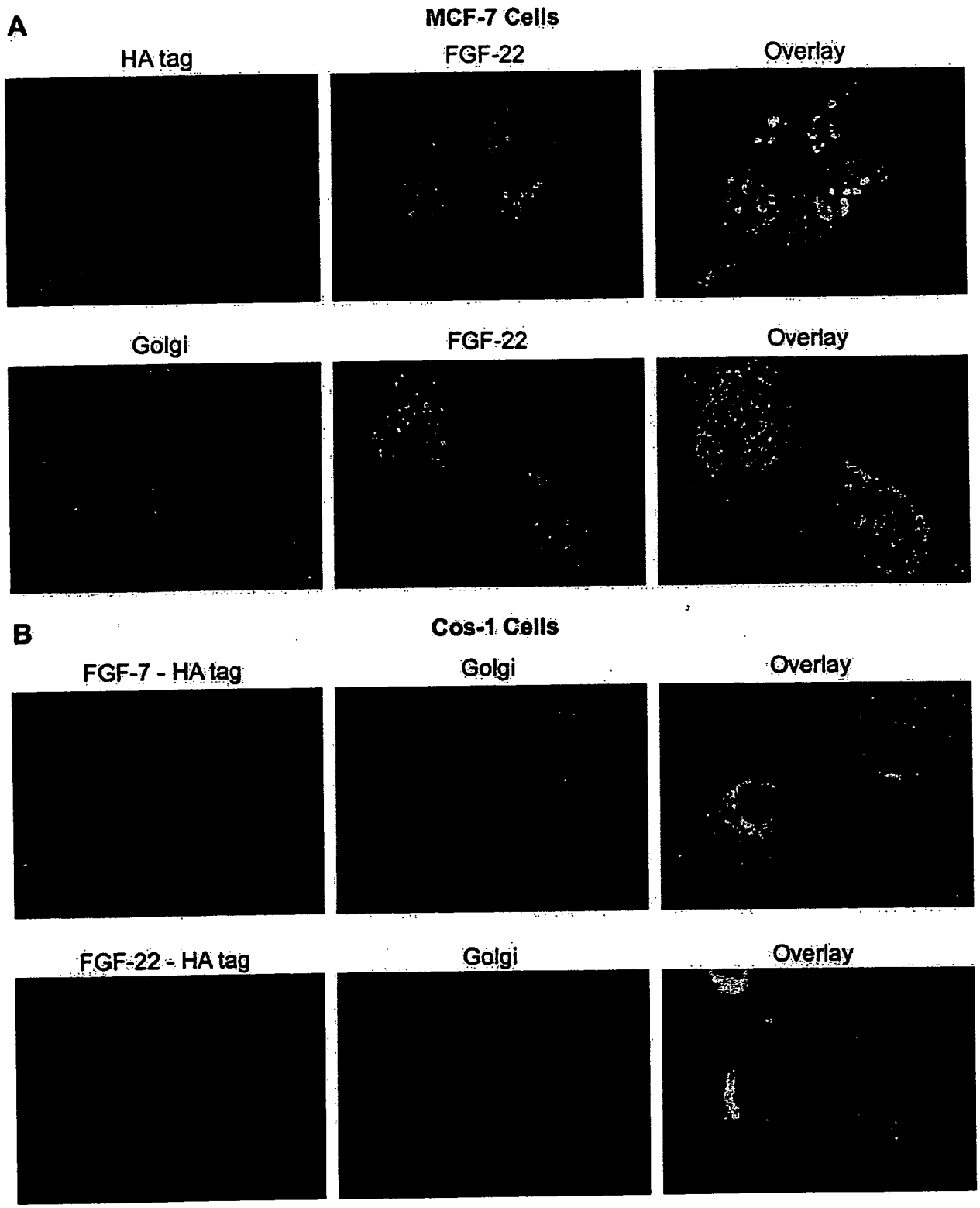


Fig. 2. FGF-22 localisation in transfected cells. (A) MCF-7 cells transfected with HA-tagged FGF-22 and incubated with antibodies to the HA epitope (red, top). Golgi 58-K protein (red, bottom) or FGF-22 (green) highlight the specificity of the FGF-22 antiserum (colocalisation with HA tag and absence of staining in untransfected cells and reveal nuclear localisation of FGF-22 protein. (B) COS-1 cells transfected with HA-tagged FGF-22 or -7 were incubated with antibodies to the HA epitope (red, left column) or Golgi 58-K protein (green, middle column). Individual and overlay images reveal an association of both FGF-7 and FGF-22 with the Golgi network.

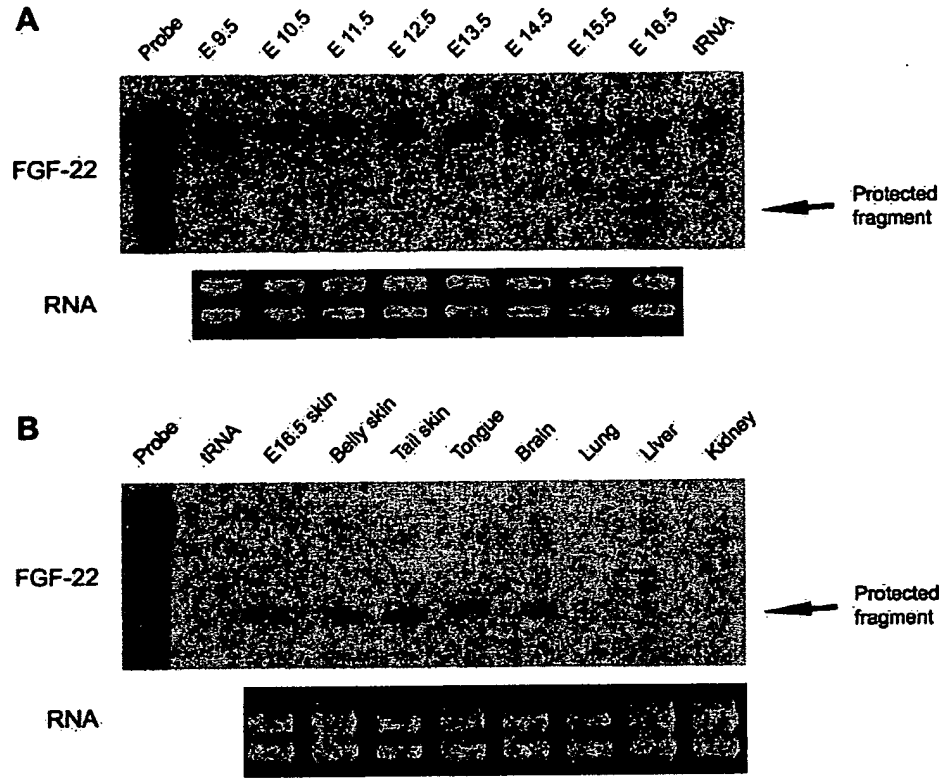


Fig. 3. FGF-22 is only detectable at later stages of development and is specifically expressed in the skin, tongue, and brain. Total cellular RNA was isolated from whole embryos (10 μg per lane; A) or from different adult murine tissues and organs (20 μg per lane; B). tRNA (20μg) was used as a negative control. Hybridization probe (1000 cpm) was loaded in the lanes labeled "probe" and used as a size marker. Thus, the upper bands running at the same height as the band in the probe lane represent small amounts of undigested probe. Each RNA (1 μg) of sample was loaded on a 1% agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays.

fectAMINE was used according to the manufacturer's instructions (Invitrogen, Paisley, UK). Primary human keratinocytes were isolated from foreskin biopsies [13] and cultured in keratinocyte serum-free medium (Life Technologies, Basel, Switzerland) (containing supplements according to the manufacturer's manual). Cells were transiently transfected using Effectene (Qiagen, Crawley, UK) after the third passage.

Cell lysis and immunoblotting

COS-1 cells were lysed directly in 2× Laemmli buffer and proteins were separated by SDS–PAGE (15% gel) before semidry transfer to nitrocellulose membranes. Membranes were incubated with the primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Antibody-binding proteins were detected by enhanced chemoluminescence (ECL) detection (Amersham Biosciences, UK). The following antibodies were used: mouse monoclonal antibody directed against the influenza virus hemagglutinin epitope (Clone 12CAS; Cancer Research UK Monoclonal Antibody facility); rabbit polyclonal antiserum (generated by Harlan Sera-Lab Ltd., Loughborough, UK) directed against the peptide GRRTRRHQLSTHFLPVLVSS (generated by the Cancer Research UK peptide synthesis laboratory), corresponding to amino acids 143–162 of murine FGF-22; and an FGF-7 specific antiserum that recognises the glycosylated and nonglycosylated forms of FGF-7 [14], but not FGF-22. Secondary antibodies for Western blots were from Promega (Madison, WI) and DAKO (Denmark).

Immunofluorescence

For immunofluorescence, cells were grown and transiently transfected on glass coverslips, prior to fixation with a 1:1 mixture of methanol/acetone for 10 min at −20°C. Cells were blocked for 1 h in PBS containing 0.2% fish gelatin, and then incubated with primary (1 h) and secondary (30 min) antibodies (diluted in PBS containing 0.1% Tween-20) at room temperature. The primary antibodies were as for Western blotting as well as a mouse monoclonal directed against Golgi 58-K protein (Sigma, Poole, UK); secondary antibodies were either anti-mouse IgG2B-Texas Red, anti-mouse IgG1-FITC and -Texas Red, or anti-rabbit IgG-FITC (Jackson ImmunoResearch, West Grove, PA).

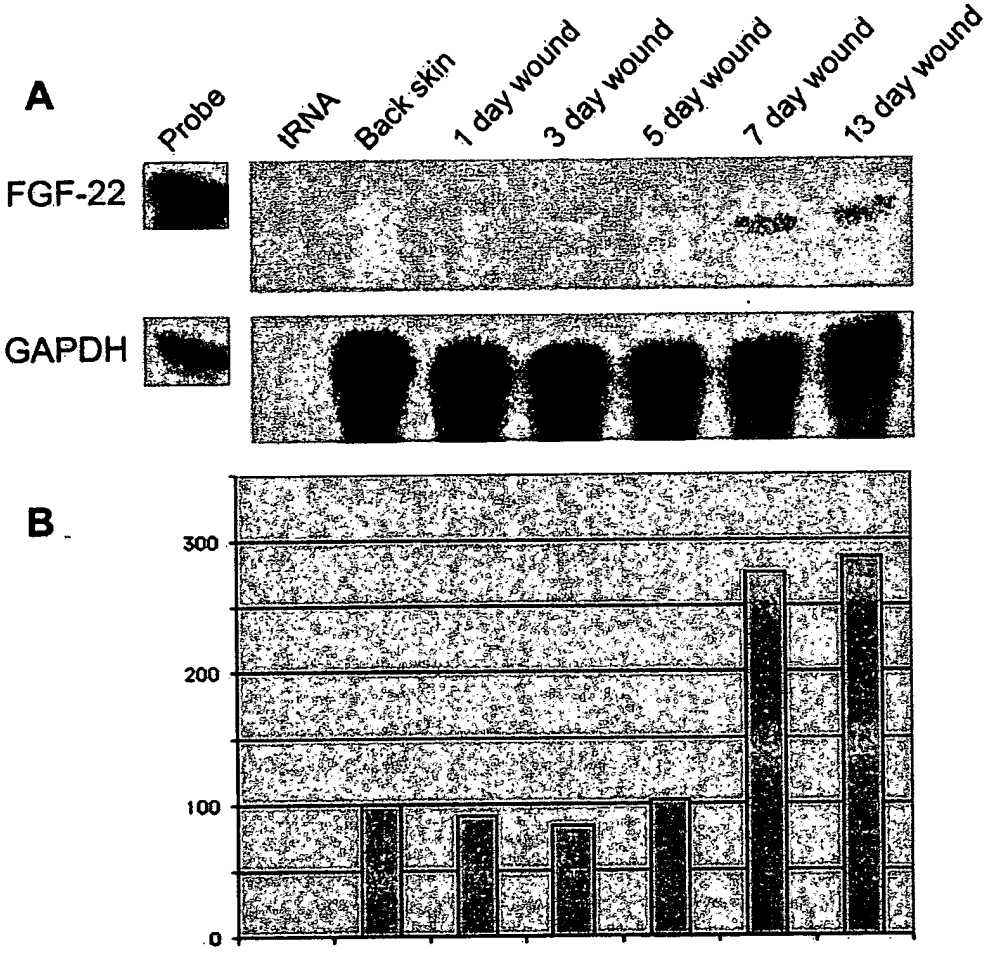


Fig. 4. FGF-22 expression increases at later stages of cutaneous wound repair. (A) total cellular RNA samples (20 μg) from nonwounded (back skin) and wounded skin (wound) were analysed by RNase protection assay for the presence of FGF-22 mRNA. To control for equal loading, samples were hybridised with both FGF-22 and GAPDH antisense riboprobes tRNA (20 μg) was used as a negative control. Each hybridization probe (1000 cpm) was loaded in the lane labeled "probe" and used as a size marker. (B) The band intensities of the RNase protection assays were quantified by phosphorimaging using Imagequant (Molecular Dynamics), normalised to the GAPDH signal, and are expressed as a percentage of the expression level in non-wounded skin. The graph shows results from a representative RNase protection assay. The result was reproduced in a second RNase protection assay using RNAs from an independent wound healing experiment.

RNA isolation and RNase protection assay

A partial murine FGF-22 cDNA (nucleotides 99–280 of the published sequence) was amplified by RT–PCR from cDNA of mouse wounds, cloned into the transcription vector pBluescript $SK^{II(+)}$ (Stratagene, La Jolla, CA) and used as a template for RNase protection assays. Total cellular RNA was isolated from whole embryos or from different adult murine tissues and organs as described by Chomczynski and Sacchi [15]. RNase protection assays were carried out according to Werner et al. [6], with 20 μg total cellular RNA used for each sample and 20 μg tRNA used as a negative control. A 120-bp fragment corresponding to nucleotides 566–685 of the mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was also used as a loading control. One thousand counts per minute of the hybridization probe was used as a size marker. As an additional control for RNA integrity and concentration, 1 μg of each RNA sample was loaded on a 1% agarose gel and stained with ethidium bromide.

Wound healing studies

Two independent wound-healing experiments were performed. For each experiment, four full-thickness excisional wounds (6 mm diam., 3–4 mm apart) were generated on the back of 20 BALB/c mice (8–12 weeks of age) as described by Werner et al. [8]. At different time points after injury (1 to 13 days), animals were euthanized and the complete wounds including 2 mm of the wound margins were isolated. At each time point, the tissue from four animals was combined, immediately frozen in liquid nitrogen, and used

for isolation of total cellular RNA (see above). A similar amount of skin from nonwounded animals served as control.

In situ hybridization

Nonwounded embryonic (E16.5) and adult back skin, as well as complete full-thickness excisional wounds (day 15 after wounding), were fixed overnight at 4°C in 4% paraformaldehyde in PBS. They were subsequently incubated in 15% sucrose in PBS for 4 h at 4°C and frozen in tissue-freezing medium. Sense and antisense riboprobes were generated using T3 or T7 RNA polymerases and digoxigenin-labeled-UTP. Frozen sections (15 μm) from the nonwounded skin and from the middle of 15-day wounds were hybridized as described by Wilkinson and Nieto [16].

Results and discussion

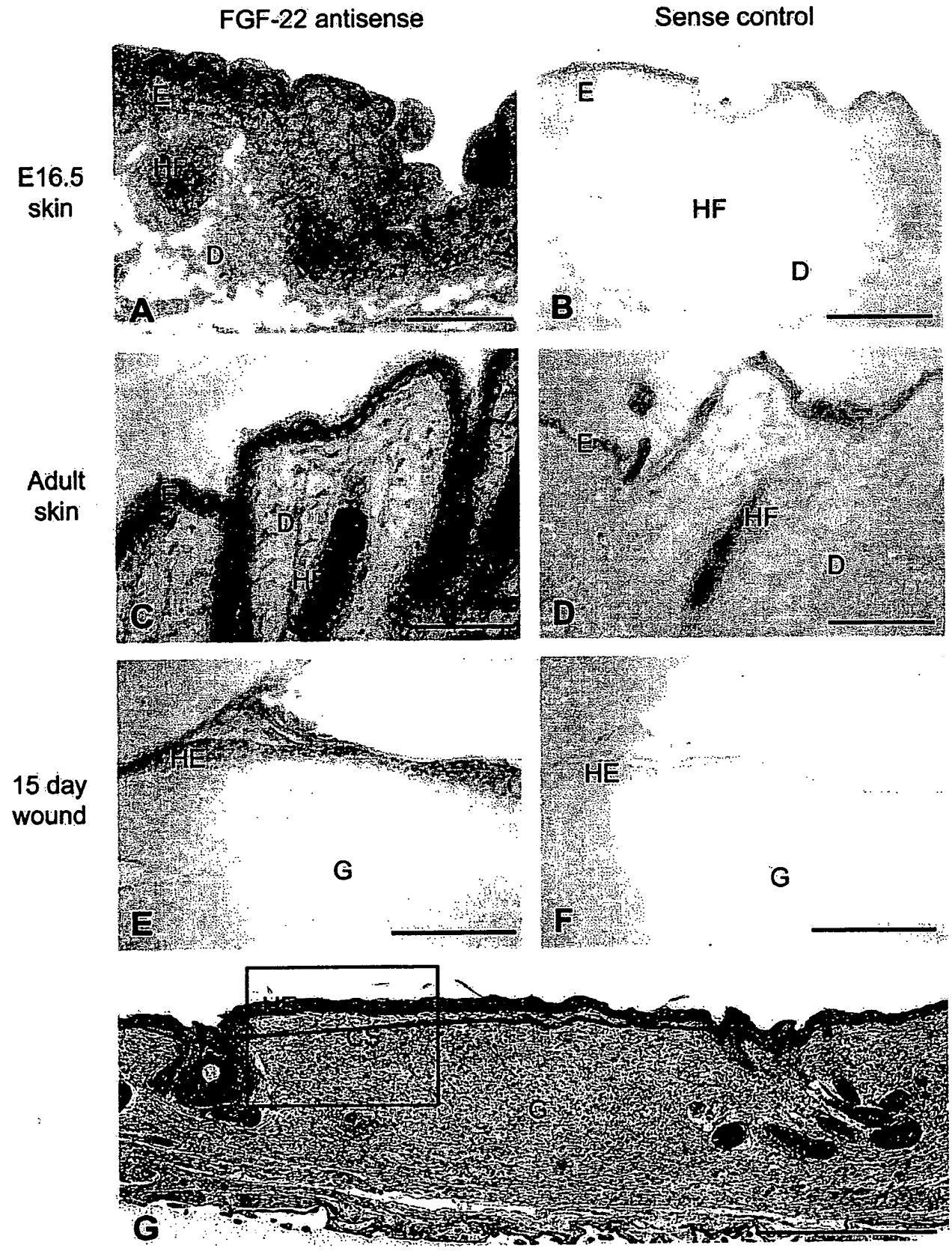
FGF-22 is cell associated in transfected mammalian cells

To characterise the properties of FGF-22, we first attempted to obtain the complete coding sequence by RT–PCR with RNA of normal and wounded mouse skin. A fragment of the expected size was amplified and cloned from 10-day wound cDNA using the primers described in experimental procedures. Full-length sequencing of the cDNA in both orientations revealed that it is identical to the sequence recently published by Nakatake et al. [12; GenBank accession number AC004449]. The cloned cDNA includes a predicted open reading frame of 486 nucleotides that should encode a protein of 162 amino acids with a calculated molecular mass of 19 kDa. In contrast to FGF-7 and FGF-10, the protein lacks a consensus sequence for N-glycosylation, a feature of most secreted FGFs.

To initially characterise the protein, it was transiently expressed in COS-1 cells as a fusion protein that includes an 18-amino-acid carboxyterminal epitope tag of the influenza virus hemagglutinin (HA). As a control, HA-tagged murine FGF-7 was expressed in the same cell type. Western blotting of lysates from COS-1 cells transfected 24 h previously with constructs for either HA-tagged mFGF-7 or -22 revealed bands of the expected sizes (approximately 26 and 23 kDa, respectively), with no product in cells transfected with the empty control vector (Fig. 1A). Expression of mFGF-22 protein was confirmed by Western blots using a rabbit polyclonal antiserum generated against a peptide corresponding to the final 20 amino acids of the murine FGF-22 protein (Fig. 1B). This antiserum showed no cross-reaction with FGF-7, as would be expected based on amino acid sequence differences. Despite several attempts, no FGF-22 could be detected in the conditioned medium of transfected COS-1 cells. On the basis of sequence information, Nakatake et al. [12] suggested that the nascent FGF-22 protein contained a putative signal peptide, though this was not experimentally addressed. The size of the detected protein could correspond to the expected size of the fusion protein including the signal peptide; however, the presence of an HA tag has been observed to increase the apparent molecular weight of other tagged proteins (S. Werner, unpublished observations). Thus, processing of the signal peptide cannot be excluded. To further investigate this apparent lack of secretion, COS-1 cells were transiently transfected with the following constructs: HA-tagged FGF-22, non-tagged FGF-7, and an additional control construct encoding a 35-kDa tagged protein known to be retained in the cytoplasm (Fig. 1C and D). FGF-22 could not be detected in conditioned medium, even after a 50-fold concentration of the supernatant using Centricon 10 concentrators (Fig. 1C). Since FGFs are often bound to glycosaminoglycans at the cell surface, 0.005 U/ml heparin was added to the culture medium after transfection. However, the presence of heparin did not lead to the appearance of FGF-22 in the conditioned medium (Fig. 1C). By contrast, FGF-7 was efficiently secreted, as demonstrated by Western blot analysis of the conditioned medium of FGF-7-transfected COS-1 cells using a FGF-7-specific antiserum, which does not cross-react with FGF-22 (Fig. 1D). The presence of an HA tag makes no difference to the secretion properties of either FGF-7 or FGF-22 (data not shown). To ensure that these differences in secretion were not the result of the cell type used for transfections, we transfected primary human keratinocytes with either tagged or untagged FGF-7 or FGF-22 expression constructs. These studies confirmed that, while both proteins were expressed in these cells, only FGF-7 was secreted (data not shown). Thus, our results revealed markedly different secretion properties between FGF-7 and FGF-22. Poor secretion was also observed for FGF-10, but partial secretion was observed after the addition of heparin to the culture medium [17].

Subcellular localisation of FGF-22 protein

To further investigate the localisation of FGF-22 protein within the cell, we used immunofluorescence on cells transiently transfected with HA-tagged FGF-22 (Fig. 2). Costaining with antibodies to FGF-22 and the HA tag confirmed the specificity of the anti-FGF-22 polyclonal rabbit antiserum (Fig. 2A, top). However, the two cell lines used showed different subcellular localisation of the protein. Surprisingly, in MCF-7 cells (human breast adenocarcinoma), FGF-22 seemed to be localised predominantly to large nuclear bodies (Fig. 2A), characteristic of the nucleolar localisation seen with some other FGFs, such as FGF-3 and FGF -1 and -2 [reviewed in 18,19]. In COS-1 cells (monkey kidney, fibroblast-like), FGF-22 was reticular and perinuclear, as expected for association with the ER/Golgi network and similar to the subcellular localisation of FGF-7 (Fig. 2B). The subcellular localisation of FGF-22 in these cell types will require more extensive analysis to determine the significance of these observations (work in progress).


FGF-22 antisense
Sense control
E16.5 skin
Adult skin
15 day wound
E
HF
D
A
B
C
HE
G
F

FGF-22 is preferentially expressed in the skin and in the brain

To determine potential sites of action of FGF-22 we first determined its expression during embryonic mouse development. Using RNase protection assays with RNA from whole mouse embryos, expression of FGF-22 was not detected before E16.5 (Fig. 3A). In the adult mouse, mRNA encoding FGF-22 was detected in the skin, tongue, and to a lesser extent brain, but not in various other organs, including lung, kidney, and liver (Fig. 3B) and heart, stomach, intestine, uterus, skeletal muscle, and spleen (data not shown). The embryonic expression data were further supported by Western blots on tissue lysates made from the skin of E16.5–18.5 embryos, where heparin sepharose was used to concentrate heparin-binding proteins prior to loading (data not shown).

FGF-22 expression is altered during cutaneous repair

To determine a potential role of FGF-22 in the wound-healing process, we generated full-thickness excisional wounds on the backs of mice and analysed the expression of this new FGF family member by RNase protection assay and in situ hybridization. The levels of FGF-22 mRNA declined during the first days after wounding and remained low until day 5 after injury. However, mRNA expression increased above basal levels at day 7 after wounding and remained elevated until day 13, when the wounds were fully healed (Fig. 4).

To localise FGF-22 expression in normal and wounded skin, we performed in situ hybridization with nonwounded embryonic (E16.5) and adult skin, as well as with 15-day-old wounds from adult mice. We also observed FGF-22 transcripts in hair follicle keratinocytes, as reported previously [12], and FGF-22 protein was first detected at E17.5, supportive of a role in hair follicle morphogenesis, which is beginning at around this time. In addition, in situ hybridization showed strong signals throughout the epidermis of nonwounded embryonic and adult skin (Fig. 5A and C) as well as in the hyperthickened wound epidermis (Fig. 5E). The latter finding provides a likely explanation for the increase in FGF-22 expression at late stages after injury, where a strongly hyperthickened epidermis is covering the wound. By contrast, only weak signals were observed in the dermis and the granulation tissue (Fig. 5A, C, and E).

The expression of FGF-22 in keratinocytes was unexpected since the highly homologous ligands FGF-7 and FGF-10 are expressed primarily in cells of mesenchymal origin. As FGFR2-IIIb, the common receptor for FGF-7 and FGF-10, is exclusively expressed by epithelial cells, these growth factors appear to act predominantly in a paracrine fashion. Although the receptor specificity of FGF-22 has not as yet been determined, the strong homology with FGF-7 and FGF-10 suggests that FGF-22 may also bind to FGFR2-IIIb as its major receptor. In this case, it would act in an autocrine manner on keratinocytes, a mechanism that has not been observed for FGF-7 or FGF-10. The possibility of autocrine stimulation undoubtedly requires potent regulatory mechanisms, since the permanent presence of an autocrine loop is likely to cause uncontrolled growth of the keratinocytes. The observed lack of secretion might be part of this regulatory mechanism, since this could prevent access of the protein to its receptor at the cell surface. Further research addressing the subcellular localisation and receptor affinity of FGF-22 will hopefully give clues as to the functional significance of its intracellular retention.

In summary, our results suggest that FGF-22, an additional member of the FGF family closely related to FGF-7 and FGF-10, is involved in epidermal homeostasis and/or cutaneous wound repair. Therefore, both FGF-10 and FGF-22 might be able to compensate for a lack of FGF-7 in FGF-7-null animals, thus explaining the unimpaired wound healing in these mice. Furthermore, our results suggest that, in addition to FGF-7 and FGF-10 [reviewed in 4,20], FGF-22 could also be useful as a therapeutic tool for the treatment of impaired wound healing and possibly lesions in other epithelia. The profile of FGF-22 expression during development also suggests a potential role for FGF-22 in hair follicle morphogenesis, where it may act as a ligand for FGFR2IIIb. Grafting studies have revealed that skin lacking this receptor displays hair defects [21], so further studies of FGF-22 function using a gene knockout approach will elucidate its role in skin development and repair.

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Fig. 5. FGF-22 is expressed by keratinocytes in normal and wounded skin. (A–F) Hybridization of antisense (A, C, and E) or sense (B, D, and F) FGF-22 probes with E16.5 embryonic skin (A and B), adult skin (C and D), and 15-day wounds (E and F). The hybridizations shown in A–D were developed for 24 h; those shown in E and F were developed for 12 h. Quantitation of relative mRNA expression levels is shown in Fig. 4. The cornified squames stained in red appear below the epidermis as an artefact of the mounting process. (G) Haematoxylin/eosin stain of a full-width 15-day wound. The location shown in E and F is indicated by the box in G. CS, cornified squames; D, dermis; E, epidermis; G, granulation tissue; HF, hair follicle; HE, hyperproliferative wound epidermis. Bars represent (A–D) 100 μm; (E and F) 200 μm; (G) 1 mm.

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